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RESEARCH ARTICLE

Comparison of the Genetic Variation of Captive Ring-tailed Lemurs with a Wild Population in Madagascar

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ABSTRACT

Genetic variability among captive and wild ring-tailed lemurs (*Lemur catta*) was assessed using mitochondrial and nuclear DNA data. A 529 bp segment of mtDNA was sequenced and 9 microsatellite loci were genotyped for 286 ring-tailed lemurs. Samples were obtained from the well-studied *L. catta* population at the Bezà Mahafaly Special Reserve and from captive animals at six institutions worldwide. We found evidence of possible patrilineal contribution but the absence of matrilineal contribution from the Bezà area, and haplotypes not found in Bezà but present in Ambohimahavelona, Andringitra Massif and other unknown locations, in the sampled captive population, indicating that the founders of the captive population originated from a wide geographic range. Total genetic variation and relatedness in captive *L. catta* in the six institutions were similar in extent to that of the wild population in Bezà. Based on the diverse origins of the captive population founders our results suggest the erosion of genetic diversity in the captive population. Sampled individuals from the same institution were more closely related to each other than members of a social group in the wild. Individuals housed at different institutions were less closely related than those of different social groups at Bezà, indicating lower genetic exchange between captive institutions than between social groups in a locality in the wild. Our findings underscore the usefulness of genotyping in determining the geographic origin of captive population founders, obtaining pedigree information if paternity is uncertain, and in maximizing preservation of extant genetic diversity in captivity.

Keywords: *Lemur catta*; captivity; genetic variability; microsatellites; mtDNA sequences; conservation

INTRODUCTION

The ring-tailed lemur (*Lemur catta*) is currently classified as a species in a monotypic genus of the family Lemuridae. This species is limited to the subtropical dry and spiny forests of southern and southwestern Madagascar [Fig. 1; Mittermeier et al., 2006; Jolly et al., 2006]. Over the last 50 years the *L. catta* population has decreased significantly due to deforestation [Sussman et al., 2003; Cameron and Gould 2013] and, in some areas, increased hunting and the pet trade [Sauther et al., 2013]. Most recently, the Madagascar unit of the IUCN has elevated the conservation status of *L. catta* to 'endangered' from its previous 'near-threatened' status [Schwitzer et al., 2014].

In the wild, ring-tailed lemurs live in groups of 10 to 20 individuals including several adult males and females. As in most mammals, females are philopatric while males disperse [Sauther et al., 1999; Gould et al., 2003]. The species is cathemeral [Parga, 2011; LaFleur et al., 2014] and both arboreal and terrestrial in habit. Among Madagascar's extant lemurs, ring-tailed lemurs are the most terrestrial, spending upwards of 33% of their time on the ground [Sauther et al., 1999]. Ring-tailed lemurs, with their characteristic long black-and-white ringed tail, are iconic and very popular in zoos, as well as being the flagship species of Madagascar, often seen in advertisements throughout the island, and even on earlier currency [Jolly et al., 2006]. In 2013 the International Species Information System (ISIS) listed 2994 individuals kept at 349 institutions around the world. Since not all zoos are ISIS members and some information may not be up-to-date, this is somewhat less than the actual captive ring-tailed lemur population.

Maintaining gene diversity of both wild and captive populations is a major conservation concern as it has a direct bearing on fitness, future adaptive potential and survival of species. However, population level genetic assessment of free ranging mammals is often difficult due to biological, logistic, and political constraints and effective addressing of threats to genetic loss is extremely challenging. While genotyping captive populations is more straightforward and selective breeding to maximize retention of gene diversity more actionable [Ivy and Lacy,

2012], preserving genetic diversity is one among many objectives of captive breeding [Earnhardt et al., 2001; Ballou et al., 2010]. Genetic management of captive populations targets preserving all the gene diversity of founders and maintaining genetic diversity of captive populations at levels comparable to source populations [Ballou et al., 2010], but is not without challenges [Lacy, 2013]. Genetic assessment of captive populations is an essential requirement for effective management [Witzenberger and Hochkirch, 2011]. Although an iconic species of endangered status, there have been no previous molecular assessments of the captive *L. catta* population.

The objective of this study was to evaluate the degree of genetic variation in captive ring-tailed lemurs held at six institutions worldwide, and its comparison with a wild population. Lemurs at the Bezà Mahafaly Special Reserve in Madagascar have been studied since 1987 [Sussman, 1991; Sauther et al., 1999; Gould et al., 2003; Cuozzo and Sauther, 2006; Sauther and Cuozzo, 2009; Sussman et al., 2013]. In total, the reserve is approximately 600 ha that includes an 80 ha gallery forest (Parcel 1) and a 620 ha dry deciduous and spiny forest (Parcel 2) [Axel and Mauer, 2010]. As of 2007, Parcel 1 of the reserve had a ring-tailed lemur population of around 225 [Sauther and Cuozzo, 2008], which includes all known, observed groups in and around Parcel 1. The overall population of the area, within 10 km of the reserve, is much larger, and inhabits a largely contiguous, though somewhat degraded, area. Genetic samples collected over several years from the Parcel 1 population provide the wild sample for comparison.

Genetic variability in nuclear and mitochondrial genomes was assessed by amplification and allelic characterization of microsatellite loci and sequencing of mtDNA, respectively. Microsatellites show a high degree of length polymorphism, and are commonly used as nuclear genetic markers to compare population level variation. The inclusion of sequence data from the hypervariable control region of the mtDNA molecule allows direct determination of matrilines, providing information on the origin of founder females in the captive population. The genetic

data were used to assess intra- and inter-group kinship of the captive and wild ring-tailed lemur populations.

METHODS

The wild data set consisted of 224 samples from the population at Bezà Mahafaly Reserve in southwest Madagascar, collected in 1987/1988, 1995, and 2003-2005, and a single sample from Amboasary Sud, near Berenty (240 km southeast of Bezà, Fig. 1). In Bezà, 1 to 34 samples were collected from 20 social groups (average 11.1 ± 9.5 samples per group). A total of 61 captive samples were obtained from 6 zoos in Europe, Australia and the United States (Table 1). Hair or blood samples were collected from living lemurs and 14 tissue samples were obtained from carcasses. Three drops of blood were collected on IsoCode Cards (Schleicher & Schuell) from some of the Bezà lemurs.

A standard phenol-chloroform extraction (Sambrook et al., 1989) was used to obtain genomic DNA from *L. catta* hair, blood or tissue samples. DNA extraction from the IsoCode Cards was carried out according to the manufacturer's instructions (Schleicher & Schuell). Approximately 10-100 ng template DNA was amplified in 20 µl (microsatellites) or 50 µl (mtDNA) reactions (see Pastorini et al. [2005] for details). The number of cycles and/or the annealing temperature was changed as necessary, to optimize PCR conditions for individual loci (Table 2).

A segment of mtDNA comprising the 3' end of the $tRNA^{Pro}$ gene and the 5' end of the control region was amplified and sequenced, using the primer pair LcProF (5' ctcaccttcaacacccaaagc 3') and LcDLR2 (5' gtcactaatccatcgagatgtc 3'). Detailed laboratory techniques for sequencing can be found in Pastorini et al. [2009]. All templates were sequenced in their entirety for both strands. The sequencing data were aligned with Sequencher™ 4.2.2 (Gene Codes Corporation) and analyzed with PAUP 4.0b10 [Swofford, 1999], with *Hapalemur*

griseus alaotrensis (GenBank # EU593893) as the outgroup. Neighbor-joining trees were calculated with Kimura two-parameter distance corrections and bootstrap analyses of 1000 replicates were performed to evaluate support of the branching order. Control region sequences from two other wild localities were obtained from GenBank # AF175499, AF175500 and AF175506; Yoder et al. 2000), which overlapped over 434 bp with our mtDNA data set.

A total of 10 microsatellite loci were used for this study (Table 2). However, one locus (Lc9, Pastorini et al. 2005) showed a trend to deviation from Hardy Weinberg equilibrium (p=0.093), likely due to a null allele. This locus was therefore excluded from all further analyses. The details for the remaining 9 loci are given in Table 2. The PCR products were run on an automated DNA sequencer. GeneScan software (Applied Biosystems) was used to determine allele sizes. For all loci, samples of 6 offspring and 4 known pairs of parents from a captive colony were genotyped to test for Mendelian inheritance.

For microsatellite data, CERVUS 3.0.3 [Kalinowski et al., 2007] was used to calculate observed (H_O) and expected (H_E) heterozygosities, as well as to test for deviations from the Hardy-Weinberg equilibrium. Genetic relatedness among and between groups or populations was evaluated using Relatedness 5.0.8 [Queller and Goodnight, 1989]. Pairwise relatedness between individuals was calculated against a background population frequency of 284 animals, with individuals weighted equally and frequency bias corrected by group. Two subjects from the Bezà population did not have unique multilocus genotypes and were excluded from relatedness calculations to prevent an underestimation of R values. R-values are estimates of genetic relatedness in a panmictic population. Parent–offspring, full siblings, and dizygotic twins are theoretically related by 0.5, half siblings by 0.25, and unrelated individuals by 0.0. Negative R-values occur when two individuals are less related than two randomly chosen individuals from the population. Differences in mean R-values between specific sets of individuals were analyzed with an unpaired, two-tailed Welch's t-test (allowing for unequal variances) and using the Satterthwaite equation to estimate the degree of freedom based on 61 captive and 223 wild animals, at a significance level of 0.05. In order to visualize the genetic

distance between individual subjects and identify hidden patterns in the data a principal component analysis (PCA) of the multilocus genotypes was conducted with GENETIX 4.05 [Belkhir et al., 2004]. The genetic structure of populations, based on microsatellite loci was inferred with STRUCTURE 2.2.2 [Pritchard et al., 2000]. The number K of populations was estimated using a burn-in period of 10,000 and 100,000 MCMC replicates, applying the admixture model and independent allele frequencies. In order to assign an individual to one or, if admixed, to several clusters STRUCTURE calculated the estimated membership coefficients Q for each individual in each cluster.

RESULTS

mtDNA

A total of 11 haplotypes (A to K) were found (GenBank # EU593882-EU593892) among the 258 ring-tailed lemurs successfully sequenced for the 529 bp mtDNA fragment. Due to low DNA quality 28 samples from Bezà could not be sequenced. Variation among haplotypes involved 25 polymorphic nucleotide positions, consisting of 23 transitions and 2 transversions. Haplotypes A, B and C were only found in the Bezà Mahafaly population (Table 3). Haplotype K was unique to the single individual from Amboasary Sud. The remaining 7 haplotypes were found in the captive population.

All ring-tailed lemurs housed at the Duke University Primate Center (DUPC) had haplotype F, which was not found in any of the other captive colonies sampled (Table 3). Haplotype G was discovered exclusively at Zürich Zoo and haplotype J was unique to Bristol Zoo. Haplotype D was found at Zürich and Emmen Zoos. All animals at Mulhouse Zoo had haplotype E, which was also present at Perth Zoo. Haplotype H occurred in lemurs housed in the zoos at Zürich, Bristol and Perth. Two ring-tailed lemurs housed at Bristol and Perth Zoos had haplotype I.

The 3 haplotypes found in the Bezà population differed from each other by 1 - 6 base positions (bp) (0.2 - 1.1%) and from the haplotype in Amboasary Sud by 5 - 8 bp (0.9 - 1.5%). The 7 haplotypes from captivity differed from each other by 1 - 11 bp (0.2 - 2.1%). Sequence divergence between the haplotypes from captivity and the wild was 7 - 14 bp (1.3 - 2.6%). The phylogenetic tree (Fig. 2) grouped the 4 haplotypes found in the wild into one clade and the 7 haplotypes of captive *L. catta* into a second clade. However, there was not much bootstrap support (52%) for this arrangement. The grouping of haplotypes A and C, the clade containing D, F and I, as well as the clade uniting E, G and H, were the only three well-supported (92-99%) nodes in the phylogenetic tree.

Microsatellite Variation

Overall, the multilocus panel was very informative. Variation at the 9 microsatellite loci in the 286 ring-tailed lemurs from the wild and in captivity is summarized in Table 4. Allelic diversity ranged from 7 to 20 alleles per locus (mean = 11.78 ± 3.67 SE). In the wild, average observed heterozygosity (H_O) was 0.76 ± 0.10, which was not significantly different from the expected average heterozygosity (H_E = 0.78 ± 0.05). In captivity, H_O was 0.71 ± 0.13, which was significantly lower than H_O in the wild ($P < 0.05$, t-Test, paired, one-tail). Calculation of H_E in captivity was not appropriate due to population structure across regions and admixture of founders (Table 4). In the Bezà population (N = 224) no loci differed significantly from Hardy-Weinberg equilibrium. With two exceptions in the Bezà population, all individuals had unique multilocus genotypes. Since the probability for two lemurs in our wild data set to have identical genotypes was only P= 3.49E-11, the two adult males must have been identical twins.

A total of 106 alleles were found in the genotypes of the 286 individuals at the 9 loci. 68 alleles (64%) occurred in both wild and captive lemurs. Of the remaining 38 alleles, 14 were present only in captive lemurs and 24 alleles were exclusive to the wild *L. catta*. The Bezà

population had 23 unique alleles (Fig. 3). In captivity alleles limited to a single institution were found in Mulhouse (N = 3), Bristol (N = 1) and Perth Zoos (N = 2).

Average genetic relatedness R among the lemurs kept at an institution was R = 0.33 ± 0.04 (ranging from 0.12 at Bristol to 0.51 at DUPC), which was significantly higher ($t(61) = 2.29$, $p = 0.026$) than average relatedness within social groups at the Bezà Mahafaly Reserve (R = 0.10 ± 0.01). In contrast, individuals housed at different zoos (R = 0.01 ± 0.02) were significantly less closely related ($t(92) = -2.23$, $p = 0.028$) than lemurs of different social groups at Bezà (R = 0.06 ± 0.01). Average relatedness within the sampled individuals from the captive population (R = 0.09 ± 0.02) was very similar ($t(92) = -0.48$, $p = 0.66$) to that among all lemurs from Bezà (R = 0.10 ± 0.01). Average relatedness between wild and captive ring-tailed lemurs was very low (R = –0.11 ± 0.01).

Genetic similarity of wild and captive lemurs based on multilocus microsatellites was visualized using a PCA. The first two PC's explained 5.01% and 4.15% of the variability across individuals in the data and mostly separated wild from captive (left and right on Fig. 4). However, one lemur from Bristol Zoo and a few from Zürich Zoo, grouped among the wild ring-tailed lemurs. *L. catta* kept at DUPC segregated slightly apart from the other captives. Animals from Zürich, Perth and Mulhouse Zoos clustered together. One lemur from Mulhouse Zoo (JP28) grouped far apart from all other ring-tailed lemurs with 3 animals (JP253, JP254, JP255) placing intermediate between JP28 and the remaining *L. catta* (Fig. 4).

The number of populations (K) was estimated by running simulations with values for K from 1 to 13. Posterior probabilities (ln Pr(X/K)) reached a plateau at K = 6, indicating that splitting the samples in 6 clusters represented the optimal subdivision of the data set. We assessed the average proportion of membership of each individual to the 6 inferred clusters. Three clusters (Q1, Q2 and Q3) occurred predominantly among the wild ring-tailed lemurs (Fig. 5). All ring-tailed lemurs from DUPC and most from Perth Zoo grouped into a single cluster

with the average proportion of membership being Q5 = 0.889 and Q4 = 0.794, respectively. Ring-tailed lemurs at the other 4 institutions were split between different clusters (Fig. 5).

DISCUSSION

Origin of Founder Animals

As the mtDNA haplotypes A, B and C from Bezà were absent in captivity, none of the matrilineal ancestors of the sampled captive population came from the Bezà Mahafaly Special Reserve and surroundings. It is important to note that the "reserve" population is not isolated, and is dynamic, as known (collared) individuals have been observed as far as 5 km from the reserve [Parga et al., 2012]. In fact, reports from local residents suggest that collared ring-tailed lemurs have been seen upwards of 12 km from the reserve. Thus, it is important to see Bezà as a region, rather than as a static, controlled population.

Haplotypes G and H in captive lemurs in our study were identical to GenBank sequence AF175506 [Yoder et al. 2000] in the overlapping segment, which was obtained from the Field Museum of Natural History collection and whose origin is given as Ambohimahavelona (60 km West of Bezà, Fig. 1). This makes it likely that the founder female(s), whose descendants are now living in Bristol, Zürich and Perth Zoos originated from the Ambohimahavelona area. This is especially interesting, as Ambohimahavelona is situated on the north side of the Onilahy River, not far from the city of Toliara. One of its ephemeral tributaries is the Sakamena River, which forms the eastern boundary of the Bezà Mahafaly Reserve, 60 km to the southeast. Given the presumed function of Madagascar's river basins as migration routes for the island's fauna [e.g., Wilmé et al., 2006] we would expect that there would be some genetic similarity between the Ambohimahavelona individual and the Bezà population, given their proximity and the distances that ring-tailed lemurs are known to migrate.

The fact that the Ambohimahavelona individual and the Bezà population segregate suggest that there was no gene flow.

Haplotype E, which we found in 22 *L. catta* housed at Mulhouse and Perth Zoos, was closely related to two GenBank sequences, differing from them by only 2 bp. The sequences AF175499 and AF175500 were collected by Yoder et al. [2000] from ring-tailed lemurs in the Andringitra Massif (240 km Northeast of Bezà, Fig. 1). Thus, the founding females of Mulhouse and Perth Zoo populations may have originated from the environs of Andringitra. The origins of the other 4 matrilines (D, F, I, J) remain unknown.

Genetic Description of the Captive Population

Microsatellite data indicated that the captive and the wild Bezà population were genetically divergent. Of all alleles found in this study 23% occurred only in the wild while 13% were only found in the captive population. The remaining 64% of alleles were present both in the wild and in captivity.

DUPC: The ring-tailed lemurs at DUPC seemed to be the most genetically distinct of all captive institutions. Animals at DUPC had a unique mtDNA haplotype found in no other of the five studied captive colonies. However, based on institutional records, the matrilines of all animals sampled from DUPC can be traced back to one female (studbook #170) brought to DUPC from St. Louis Zoo (USA). Nothing is known of her birth or her parents (ISIS, 1997). Principal component analysis grouped the DUPC lemurs apart (Fig. 4) and admixture analyses clearly assigned them to a single cluster ($Q5 = 0.889 \pm 0.04$, Fig. 5). Compared to other zoos, average relatedness among DUPC animals was very high and they had the lowest number of alleles (Fig. 3). The 10 samples analyzed in our study were from two mating pairs and their 6 offspring, which may partly account for the high relatedness. However, ring-tailed lemurs kept at other institutions are also very likely to be closely related to each other. A study of 73 *L. catta* kept at DUPC found some to suffer from inbreeding depression [Charpentier et al., 2008].

Mulhouse: Interestingly, principal component analysis grouped 4 *L. catta* from Mulhouse Zoo far apart from all other ring-tailed lemurs (Fig. 4). The most distinct animal JP28 is the father of the 3 individuals JP253, JP254 and JP255 grouped between him and the other ring-tailed lemurs. The parents of JP28 were captive born, one in France and one in Northern Ireland. Origins of the grandparents on JP28's sire's side and all 4 great-grandparents from the dam's side are unknown (ISIS, 1997). His mtDNA haplotype is the same as found in all other *L. catta* at Mulhouse. Based on these findings we suggest that the patriline of JP28 may have originated from a location remote from Bezà, Amboasary Sud, Ambohimahavelona and Andringitra. In admixture analysis all 10 Mulhouse lemurs had a large proportion assigned to cluster Q6 (Q6 = 0.277 - 0.737). In addition, 6 animals had a considerable proportion of cluster Q4 (0.239 - 0.500) while the other 4 animals had cluster Q5 (0.149 - 0.339). Q4 was predominant at Perth Zoo and Q5 was found in all animals at DUPC and in some animals at Zürich Zoo (Fig. 5). The samples of the 4 lemurs assigned to Q5 were collected in 1976 from an adult female and her 3 offspring. The other 6 samples assigned to Q4 were collected in 1997 from 2 breeding females, 1 breeding male (JP28) and their 3 offspring.

Zürich Zoo: Principal component analysis positioned 4 ring-tailed lemurs from Zürich Zoo among the animals from the Bezà population, indicating that the Zürich population might have an ancestor from the environs of Bezà (Fig. 4). Admixture analyses revealed 8 animals with a main proportion of cluster Q6 (Fig. 5), 2 animals with a mixture of clusters Q5 and Q6, 6 animals with a majority of Q5. Three animals appeared to be admixed for Q5 and the wild cluster Q2. The latter again supports the origin of a founder of the Zürich colony from the Bezà area. This is not a surprise, as many of the lemur specimens collected in Madagascar (whether live or for museum collections) came from areas along major rivers and their tributaries, which matches Bezà's location [e.g., Buettner-Janusch and Tattersall, 1985]. The affiliation with Bezà in the nuclear but not in the mitochondrial genome, suggests a patrilineal contribution from Bezà to the Zürich population. Notably, with one exception, the animals grouping among the wild individuals in the principal component analysis are not the same as the ones showing wild

admixed genotypes. Unfortunately, no information (also no studbook numbers) was available of the origin of the Zürich population and the familial relationships of the sampled lemurs to each other.

Bristol Zoo: Principal component analysis grouped 3 animals from Bristol Zoo apart from the other captive ring-tailed lemurs and the fourth animal JP449 was placed among the wild *L. catta* (Fig. 4). Three animals had high proportions of membership in the wild clusters Q1 - Q3 (Fig. 5). JP449 had a high proportion of membership in cluster Q5 (0.414), while the other 3 lemurs from Bristol Zoo exhibited more of Q4 (0.390 - 0.712). Notably, all ring-tailed lemurs at Perth Zoo have a high proportion of Q4, possibly originating from their founder from Bristol Zoo. There were 3 different mtDNA haplotypes among the 4 ring-tailed lemurs at Bristol, one of which was found in no other lemur sampled for this study. One female from Bristol Zoo is known to be wild-born in Madagascar (ISIS, 1997). Her mtDNA haplotype was H, which was well represented in the captive population (Perth and Zürich Zoos, Table 3) and which was assigned to the environs of Ambohimahavelona in the wild. Microsatellite and mtDNA data indicated high genetic variation of these 4 animals at Bristol Zoo, which was also reflected in Bristol having the lowest average relatedness among the captive institutions sampled. This is due to having sampled one founder female (JP450) and two other completely unrelated males (JP448 and JP449) born at different zoos. Only the fourth ring-tailed lemur (JP447) was related with JP448 being his father, and JP450 being the great-grandmother (ISIS, 1997).

Emmen: Admixture analysis indicated a high proportion of the wild clusters (Q1 + Q2 + Q3 = 0.432, Fig. 5), indicating that there was some genetic contribution from the Bezà area. However, with a sample size of only one animal nothing much can be said about Emmen Zoo.

Perth Zoo: Since the ring-tailed lemurs at Perth Zoo were kept in a multi-male/multi-female group, paternity of the offspring was not clear (ISIS, 1997). With 3 mtDNA haplotypes the Perth Zoo population was as variable as Zürich Zoo with regard to mtDNA. The captive population at Perth Zoo was founded with two breeding pairs, one from Bristol Zoo and one

from Naples Zoo (Italy). Four founder animals represent a maximum of 4 mtDNA haplotypes. As the haplotypes from males are not inherited they will not be represented from the F1 generation on. Haplotypes E and H were found in 14 sampled lemurs. Therefore, those haplotypes were introduced by the two founder females from Bristol and Naples. The third haplotype I was found only in only one male at Perth Zoo. Therefore, he must be one of the two founder males. Haplotype I was also present in Bristol, indicating that he might have come from Bristol. Admixture analyses assigned most Perth animals mainly to one cluster (Q4 = 0.846 ± 0.075). One female (JP645) was different from all the others with Q2 = 0.452 and Q5 = 0.220 (Fig. 5). Her mtDNA haplotype was H, which was only found in one more male at Perth Zoo but was also found at Bristol and Zürich Zoos. Given her distinct nuclear DNA genotype, JP645 represents one of the founder lineages and may be a founder female. The only other animal with haplotype H was her only sampled offspring in the population. The most common mtDNA haplotype at Perth Zoo was E (12 out of 15 animals). Therefore the second founder female of the Perth population had a greater representation than JP645. Since haplotype E was not found in the 4 animals from Bristol Zoo, she may have originated from Naples Zoo. However, we do not have samples from all the animals at Bristol Zoo nor do we have any samples from Naples Zoo, so this is not definitive.

Unfortunately, little information was available on the captive ring-tailed lemur population founders' capture locations (ISIS, 1997). With microsatellite data from only two locations in the wild (Bezà Mahafaly and Amboasary-Sud) and mtDNA data from four locations (Fig. 1), our assessment of the founders' possible origins is very preliminary. There are no published population genetic studies of sufficient coverage and detail to establish population structure across a species distribution for *L. catta* or other lemur species. More extensive genetic sampling of wild populations across the distribution range would provide more conclusive evidence on the origins of founder animals and patterns of genetic structure in the wild.

Genetic Structure of the Captive Population

Sequence divergence values and phylogenetic analyses did not identify any of the groups as being highly divergent. This finding is in agreement with an earlier study on ring-tailed lemurs using mtDNA sequence data of the control region and the cytochrome *b* gene to assess level of genetic divergence [Yoder et al., 2000].

The number of alleles (Fig. 3) as well as average genetic relatedness among all sampled individuals in captivity was similar to that in the wild population at Bezà. Thus the genetic variation in captivity among the six institutions in three management regions (Australian, European and North American) equaled that of a wild population. A genetic study using microsatellites suggested that a bottleneck may have occurred among *L. catta* in southwestern Madagascar in the recent past [Parga et al., 2012], which would result in loss of genetic variation. Therefore, genetic diversity in the Bezà population may have been reduced by a bottleneck. Considering that the captive population sample included mtDNA from at least two distinct populations 337 km apart from each other (Ambohimahavelona and Andringitra Massif, Fig. 1), and nuclear contribution from more populations, higher genetic variation would be expected. Therefore, our results indicate possible erosion of genetic variability in the captive population.

Average genetic relatedness within captive colonies was higher than within social groups at Bezà. This of course is expected, as the population in and around Bezà, despite rapid habitat degradation, still displays long-distance (>10 km) migration of individuals, most often by males [Sussman, 1992; Parga et al., 2012], but also by females [Parga et al., in press]. In contrast, average relatedness between captive institutions was much lower than relatedness between groups in the wild. This suggests that genetic exchange between the sampled captive institutions was lower than between the observed social groups in the wild. Despite the captive population having the genetic variation of a wild population, the genetic structure and connectivity of the captive population was clearly different from that in the wild.

CONSERVATION AND MANAGEMENT IMPLICATIONS

Having genotyped only 61 animals out of about 3000 ring-tailed lemurs living in captivity, sample size is obviously not sufficient to give a conclusive picture of the genetic variation of the captive population. While genetic variation of the animals sampled across six institutions on three continents equals that of the wild population at Bezà Mahafaly Reserve, genetic exchange between institutions was found to be low. This despite the ring-tailed lemurs being part of regional breeding programs with frequent exchanges that aim to minimize mean kinship and equalize (presumed) founder relationships. Genetic theory predicts that a population consisting of several small isolated groups will have greater genetic diversity, less inbreeding, and less genetic adaptation to captivity than a single large population [Lacy, 1987; Margan et al., 1998]. On the other hand, small isolated groups are more likely to become extinct. Captive management of a species has to contend with inbreeding depression as well as outbreeding depression [Witzenberger and Hochkirch, 2011]. Judging by the total extant numbers, captive ring-tailed lemurs seem to be doing well. However, a recent study on the ring-tailed lemurs kept at DUPC [Charpentier et al., 2008] showed that loss of genetic diversity had a negative impact on the health of the animals, even affecting their survival. It is important to preserve the genetic variation at each institution, to ensure that the captive population maintains an extent of genetic variation similar to a wild population. Since genetic variability of the captive population as a whole is relatively high, regular exchange of ring-tailed lemurs between institutions can prevent undesired consequences from inbreeding at institution levels [Charpentier et al., 2008].

As shown in other species managed in captivity like cranes [Jones et al., 2002], vultures [Gautschi et al., 2003], horses [Bowling et al., 2003], tortoises [Russello et al., 2007], wallabies [Ivy et al., 2009], and tapirs [Gonçalves da Silva et al., 2010], we found the molecular data to be very useful in deciphering the breeding history and origin of ring-tailed lemurs, where detailed pedigrees and information of the founders' origins are not available. Genotyping all captive ring-tailed lemurs and using the results to guide ongoing exchange and breeding programs

would maximize preservation of genetic variability which is desirable for the long term conservation of *L. catta* in captivity. This in turn has important implications for any potential reintroduction of captive ring-tailed lemurs to the wild. Given the rapidly increasing threats to this now endangered species and its native habitat [e.g., Sauther et al., 2013], elucidating the geographic origin of captive lemurs may significantly benefit future conservation efforts, including potential reintroduction.

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REFERENCES

Axel AC, Maurer BA. 2010. Lemurs in a complex landscape: Mapping species density in subtropical dry forests of southwestern Madagascar using data at multiple levels. Am J Primatol 71:1-15.

Buettner-Janusch B, Tattersall I. 1985. An annotated catalogue of Malagasy primates (familes Lemuridae, Indriidae, Daubentoniidae, Megaladapidae, Cheirogaleidae) in the collections of the American Museum of Natural History. Am Mus Novit 2834:1-45.

Ballou JD, Lees C, Faust LJ, Long S, Lynch C, Bingaman L, Foose TJ. 2010. Demographic and genetic management of captive populations. In: Kleiman DG, Thompson KV, Kirk Baer C, editors. Wild Mammals in Captivity: Principles and Techniques for Zoo Management. Chicago: University of Chicago Press. p 219-252.

Bowling AT, Zimmermann W, Ryder O, Penado C, Peto S, Chemnick L, Yasinetskaya N, Zharkikh T. 2003. Genetic variation in Przewalski's horses, with special focus on the last wild caught mare, 231 Orlitza III. Cytogenet Genome Res 101:226–234.

Cameron A, Gould L. 2013. Fragment-adaptive behavioural strategies and intersite variation in the ring-tailed lemur (*Lemur catta*) in South-Central Madagascar. In: Marsh LK, Chapman CA, editors. Primates in Fragments: Complexity and Resilience, Developments in Primatology: Progress and Prospects. New York: Springer. p 227-243.

Charpentier MJE, Williams CV, Drea CM. 2008. Inbreeding depression in ring-tailed lemurs (*Lemur catta*): genetic diversity predicts parasitism, immunocompetence, and survivorship. Conserv Genet 9:1605–1615.

Cuozzo FP, Sauther ML. 2006. Temporal change in tooth size among ring-tailed lemurs (*Lemur catta*) at the Beza Mahafaly Special Reserve, Madagascar: effects of an environmental fluctuation. In: Jolly A, Sussman RW, Koyama N, Rasmimanana H, editors. Ring-tailed Lemur Biology. New York: Springer. p 343-366.

Earnhardt JM, Thompson SD, Marhevsky EA. 2001. Interactions of target population size, population parameters, and program management on viability of captive populations. Zoo Biology 20:169-183.

Gautschi B, Jacob Gl, Negro JJ, Godoy JA, Müller JP, Schmid B. 2003. Analysis of relatedness and determination of the source of founders in the captive bearded vulture, *Gypaetus barbatus*, population. Conserv Gen 4:479-490.

Gonçalves da Silva A, Lalonde DR, Quse V, Shoemaker A, and Russello MA. 2010. Genetic approaches refine ex situ lowland tapir (*Tapirus terrestris*) conservation. J Hered 101:581-590.

Gould L, Sussman RW, Sauther ML. 2003. Demographic and life-history patterns in a population of ring-tailed lemurs (*Lemur catta*) at Beza Mahafaly Reserve, Madagascar: A 15-year perspective. Amer J Phys Anthropol 120:182-194.

Ivy JA, Miller A, Lacy RC, DeWoody JA. 2009. Methods and prospects for using molecular data in captive breeding programs: An empirical example using parma wallabies (*Macropus parma*). J Hered 100:441–454.

Ivy JA, Lacy RC. 2012. A comparison of strategies for selecting breeding pairs to maximize genetic diversity retention in managed populations. J Hered 103:186–196.

ISIS (International Species Information System). 1997. Ring-tailed Lemur Studbook. Compiled with SPARKS on 6. Nov. 1997.

ISIS (International Species Information System). 2013. Species holding data for *Lemur catta* as of March 7th, 2013.

Jolly A, Sussman RW, Koyama N, Rasmimanana H, editors. 2006. Ring-tailed Lemur Biology. New York: Springer.

Jones KL, Glenn TC, Lacy RC, Pierce JR, Unruh N, Mirande CM, Chavez-Ramirez F. 2002. Refining the whooping crane studbook by incorporating microsatellite DNA and leg-banding analyses. Conserv Biol 16:789–799.

Kalinowski ST, Taper ML, Marshall TC. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol 16:1099-1106.

Lacy RC. 1987. Populations: Interacting effects of drift, mutation, immigration, selection, and population subdivision. Conserv Biol 1:143-158.

Lacy RC. 2013. Achieving true sustainability of zoo populations. Zoo Biol 32:19-26.

LaFleur M, Sauther M, Cuozzo F, Yamashita N, Jacky Youssouf IA, Bender R. 2014. Cathemerality in wild ring-tailed lemurs (*Lemur catta*) in the spiny forest of Tsimanampetsotsa National Park: camera trap data and preliminary behavioral observations. Primates 55:207-217.

Lawler RR, Richard AF, Riley MA. 2001. Characterization and screening of microsatellite loci in a wild lemur population (*Propithecus verreauxi verreauxi*). Am J Primatol 55:253-259.

Margan SH, Nurthen RK, Montgomery ME, Woodworth LM, Lowe EH, Briscoe DA, Frankham R. 1998. Single large or several small? Population fragmentation in the captive management of endangered species. Zoo Biol 17:467–480.

Merenlender AM. 1993. The effects of sociality on the demography and genetic structure of *Lemur fulvus rufus* (polygamous) and *Lemur rubriventer* (monogamous) and the conservation implications. Ph.D. thesis: University of Rochester. 166 p.

Mittermeier RA, Konstant WR, Hawkins F, Louis EE, Langrand O, Ratsimbazafy J, Rasoloarison R, Ganzhorn JU, Rajaoblina S, Tattersall I, Meyers DM. 2006. Lemurs of Madagascar. Second Edition. Washington, DC: Conservation International. 356 p.

Parga JA. 2011. Nocturnal ranging by a diurnal primate: are ring-tailed lemurs (*Lemur catta*) cathemeral? Primates. 52:201-205.

Parga JA, Sauther ML, Cuozzo FP, Jacky IAY, Lawler RR. 2012. Evaluating ring-tailed lemurs (*Lemur catta*) from Southwestern Madagascar for a genetic population bottleneck. Am J Phys Anthropol 147:21–29.

Parga JA, Sauther ML, Cuozzo FP, Jacky IAY, Gould L, Sussman RW, Lawler RR, Pastorini J. in press. Genetic evidence for male and female dispersal in wild *Lemur catta*. Folia Primatol.

Pastorini J, Fernando P, Melnick DJ, Forstner MRJ. 2004. Isolation of 10 microsatellite markers for mongoose lemurs (*Eulemur mongoz*). Mol Ecol Notes 4:67-69.

Pastorini J, Fernando P, Forstner MRJ, Melnick DJ. 2005. Characterization of new microsatellite loci for the ring-tailed lemur (*Lemur catta*). Mol Ecol Notes 5:149-151.

Pastorini J, Zaramody A, Curtis DJ, Nievergelt CM, Mundy NI. 2009. Genetic analysis of hybridization and introgression between wild mongoose and brown lemurs. BMC Evol Biol 9:32.

Queller DC, Goodnight KF. 1989. Estimating relatedness using genetic markers. Evolution 43:258-275.

Sauther ML, Cuozzo FP. 2008. Somatic variation in living, wild ring-tailed lemurs (*Lemur catta*). Folia Primatol 79:55–78.

Sauther ML, Cuozzo FP. 2009. The impact of fallback foods on wild ring-tailed lemur biology: A comparison of intact and anthropogenically disturbed habitat. Am J Phys Anthropol 140:671-686.

Sauther ML, Sussman RW, Gould L. 1999. The socioecology of the ringtailed lemur: Thirty-five years of research. Evol Anthropol 8:120-132.

Sauther ML, Cuozzo FP, Youssouf Jacky IA, Fish KD, Lafleur M, Ravelohasindrazana LAL, Ravoavy JF. 2013. Limestone cliff - face and cave use by wild ring-tailed lemurs (*Lemur catta*) in southwestern Madagascar. Madagascar Conserv Develop 8:73-80.

Schwitzer C, Mittermeier RA, Johnson SE, Donati G, Irwin M, Peacock H, Ratsimbazafy J, Rasafindramanana J, Louis Jr. EE, Chikhi L, Colquhoun IC, Tinsman J, Dolch R, LaFleur M, Nash S, Patel E, Randrianambinina B, Rasolofoharivelo T, Wright PC. 2014. Averting lemur extinctions amid Madagascar's political crisis. Science 343:842-843.

Sussman RW. 1992. Male life history and intergroup mobility among ring-tailed lemurs (*Lemur catta*). Int J Primatol 13:395–413.

Sussman RW, Green GM, Porton I, Andrianasolondraibe OL, Ratsirarson J. 2003. A survey of the habitat of *Lemur catta* in southwestern and southern Madagascar. Primate Conservation 19:32-57.

Sussman RW, Richard AF, Ratsirarson J, Sauther ML, Brockman DK, Gould L, Lawler R, Cuozzo FP. 2012. Beza Mahafaly Special Reserve: A research site in Southwestern Madagascar. In: Kappeler P, Watts D, editors. Long Term Field Studies of Primates. New York: Springer. p 45-66.

Swofford DL. 1999. PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Version 4.0b2. Sunderland: Sinauer Associates.

Wilmé L, Goodman SM, Ganzhorn JU. 2006. Biogeographic evolution of Madagascar's microendemic biota. Science 312: 1063–1065.

Wimmer B, Kappeler PM. 2002. The effects of sexual selection and life history on the genetic structure of redfronted lemur, *Eulemur fulvus rufus*, groups. Animal Behav 64:557-568.

Witzenberger KA, Hochkirch A. 2011. Ex situ conservation genetics: a review of molecular studies on the genetic consequences of captive breeding programmes for endangered animal species. Biodivers Conserv 20:1843-1861.

Yoder AD, Irwin JA, Goodman SM, Rakotoarisoa SV. 2000. Genetic tests of the taxonomic status of the ring-tailed lemur (*Lemur catta*) from the high mountain zone of the Andringitra Massif, Madagascar. J Zool, London 252:1-9.

FIGURES

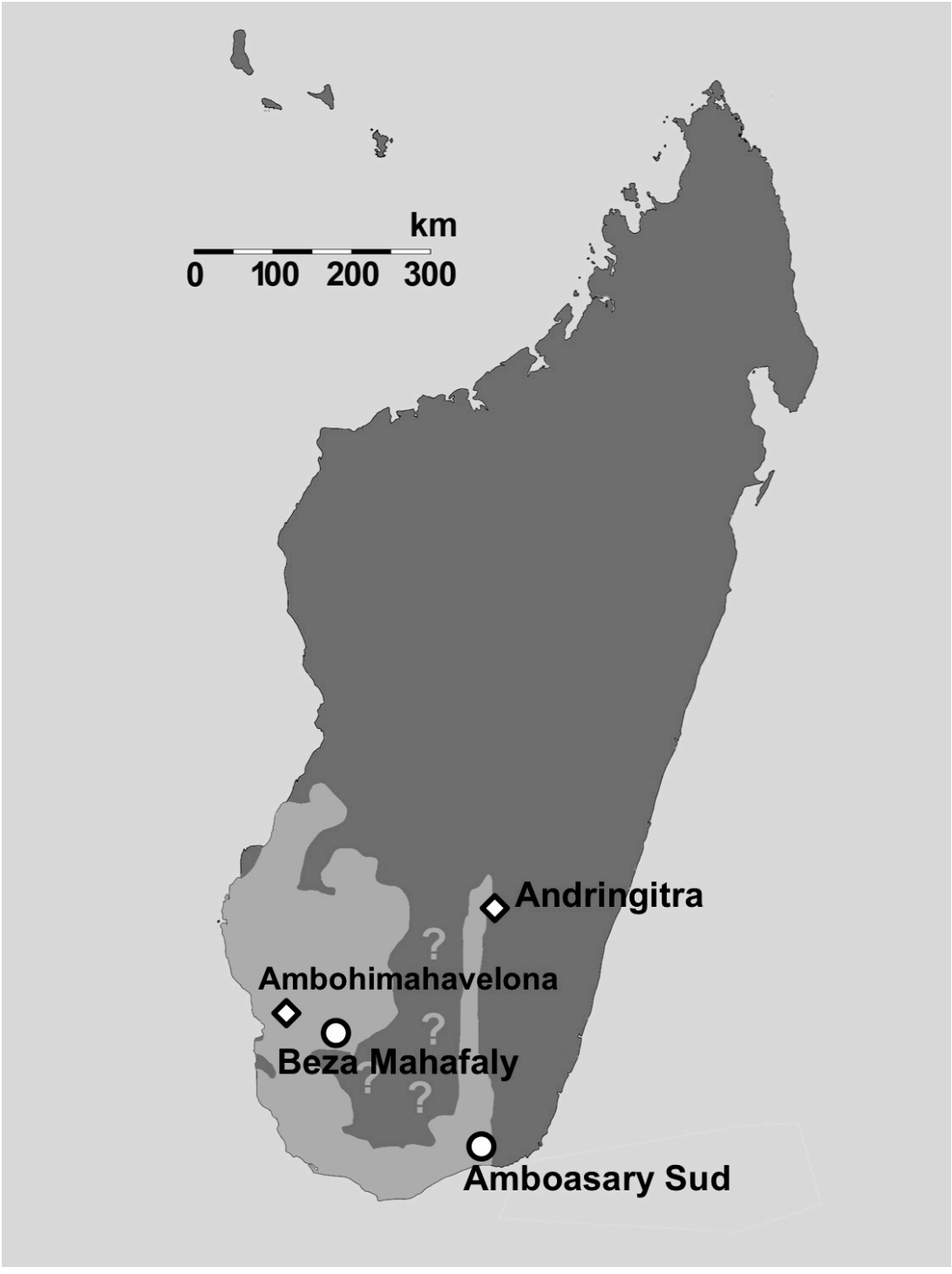

Fig. 1. Map of Madagascar depicting the ring-tailed lemur's distribution in the South [Mittermeier et al. 2006] and showing the two sampling locations (circles) and two locations of sequences obtained from GenBank (diamonds).

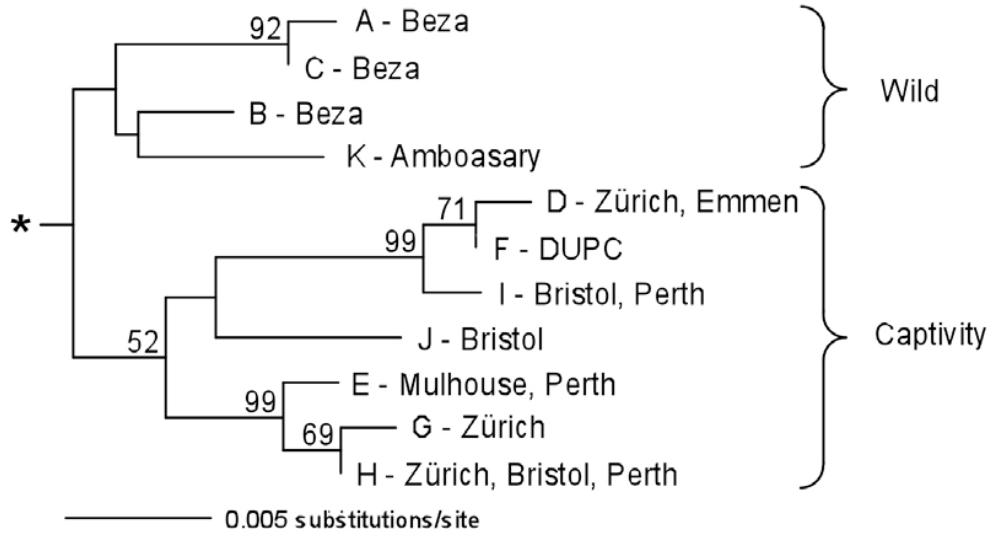

Fig. 2. Neighbor-joining tree of the 11 mtDNA haplotypes with bootstrap support values greater than 50% obtained in 1000 replicates. * The outgroup used in the analyses is not depicted.

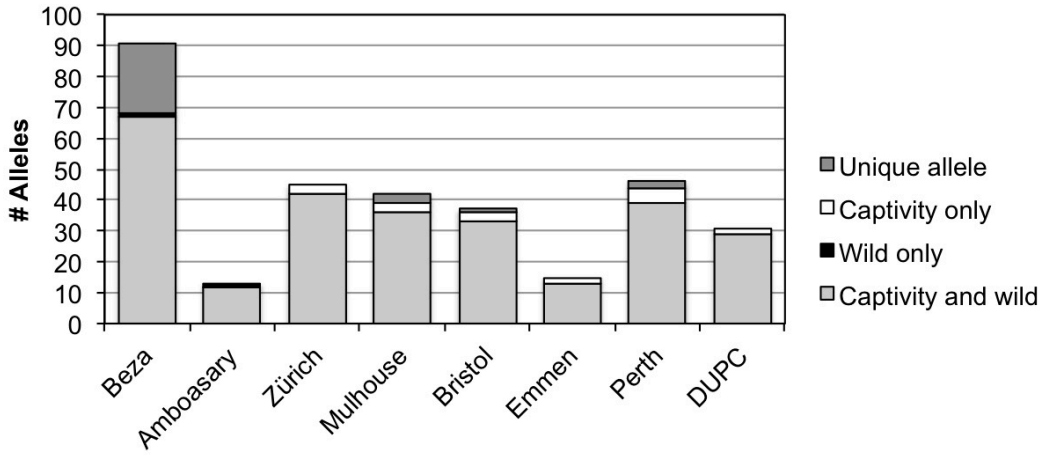

Fig. 3. Number and type of microsatellite alleles found at the two localities in the wild (Bezà and Amboasary), and at each captive colony.

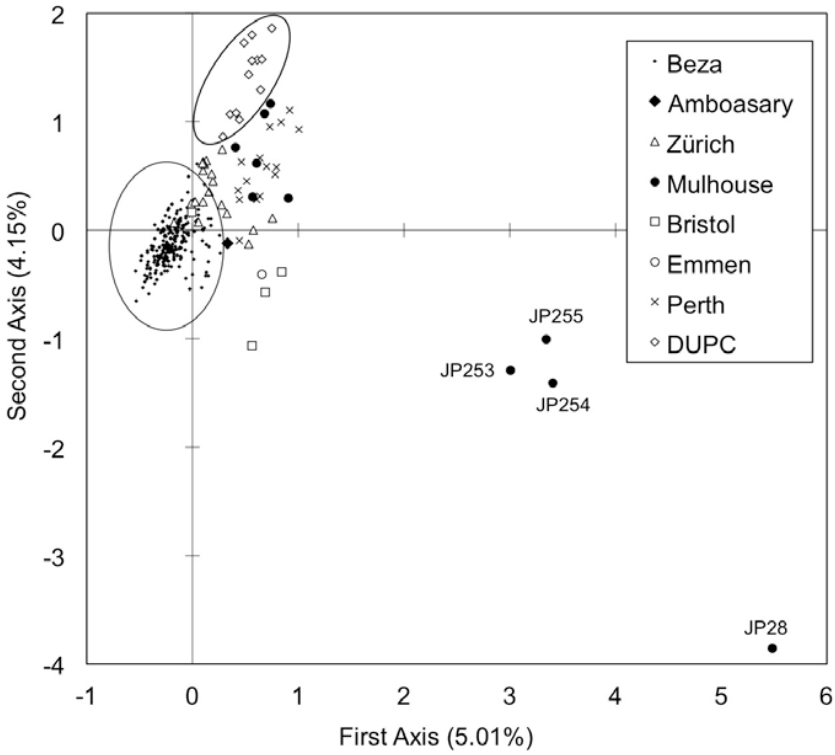

Fig. 4. Principal component analysis of individual *L. catta* genotypes. First and second axes represent the first two factorial components. Two clusters containing animals from the wild (left) and DUPC (top) as well as 4 individuals from Mulhouse (JP28, JP253, JP254, JP255) are labelled.

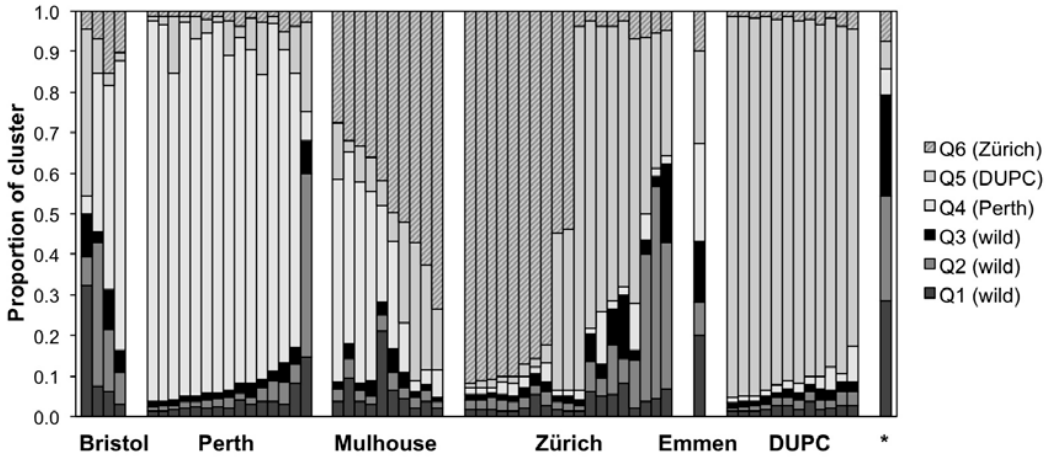

Fig. 5. Admixture analysis of captive and wild ring-tailed lemurs. Each captive animal is represented by a single vertical bar broken into K=6 segments, with lengths proportional to the estimated membership in each cluster (Q1 - Q6). * The 225 wild *L. catta* are summarized in a single bar, each cluster showing the average proportion of membership.

TABLE 1. Samples available from the wild and captivity

Origin	Locality	Year of acquisition	# Samples
Madagascar	Bezà Mahafaly Reserve	1987-2003	224
	Amboasary-Sud, near Berenty	1991	1
Europe	Zürich Zoo, Switzerland	1977-2001	19
	Mulhouse Zoo, France	1976-1997	10
	Bristol Zoo, UK	1991	4
	Emmen Zoo, Netherlands	1993	1
USA	Duke University Primate Center (DUPC)	2003	12
Australia	Perth Zoo	1995-2002	15

TABLE 2. Characteristics of the nine microsatellite loci used in this study *

Locus	Isolated from	Repeat motif in *L. catta*	T_a (ºC)	Size range (bp)	N_I	N_A	Citation
Lc5	*L. catta*	$(TC)_{15}$ TT $(TC)_6$ TG $(TC)_4$ TG $(TC)_6$	55	127-149	280	10	Pastorini et al., 2005
Lc6	*L. catta*	$(TC)_{14}$ AC $(TC)_{11}$	60	247-269	286	10	Pastorini et al., 2005
Lc8	*L. catta*	$(CA)_{14}$	55	199-218	236	10	Pastorini et al., 2005
Lc10	*L. catta*	$(AC)_{10}$ GC $(AC)_4$	55	140-168	281	11	Pastorini et al., 2005
Em7	*Eulemur mongoz*	$(GT)_4$ $(GN)_2$ GT $(GA)_{14}$	60	131-145	284	7	Pastorini et al., 2004
Em12	*E. mongoz*	$(TC)_{14}$	60	121-174	284	20	Parga et al., in press
Efr02W	*E. fulvus rufus*	$(TG)_{18}$	60	189-207	283	11	Wimmer and Kappeler, 2002
EfrL2M	*E. fulvus rufus*	$(CA)_{22}$	60	178-202	279	13	Merenlender, 1993
Pv1L	*Propithecus verreauxi*	$(GT)_{23}$	60	150-174	258	14	Lawler et al., 2001

* T_a = annealing temperature; N_I = number of individuals genotyped; N_A = number of alleles found

TABLE 3. Frequency of mtDNA haplotypes at each location

Locality	A	B	C	D	E	F	G	H	I	J	K	Total
Bezà	125	70	1									196
Amboasary											1	1
Zürich				8			8	3				19
Mulhouse					10							10
Bristol								2	1	1		4
Emmen				1								1
Perth					12			2	1			15
DUPC						12						12
All	125	70	1	9	22	12	8	7	2	1	1	258

TABLE 4. Results for the nine microsatellite loci used in this study *

Locus	Wild					Captivity		
	N_I	N_A	H_O	H_E	P	N_I	N_A	H_O
Lc5	219	9	0.790	0.803	NS	61	9	0.787
Lc6	225	8	0.636	0.657	NS	61	9	0.607
Lc8	177	7	0.734	0.758	NS	59	7	0.746
Lc10	220	10	0.809	0.794	NS	61	8	0.623
Em7	223	5	0.587	0.621	NS	61	6	0.475
Em12	223	17	0.888	0.894	NS	61	15	0.885
Efr02W	222	10	0.739	0.756	NS	61	9	0.689
EfrL2M	220	12	0.827	0.862	NS	59	10	0.864
Pv1L	198	14	0.838	0.870	NS	60	9	0.750
Average	214.1	10.2	0.761	0.779	NS	60.4	9.1	0.714

* N_I = number of individuals genotyped; N_A = number of alleles found; H_O = observed heterozygosity; H_E = expected heterozygosity; P = p-value for significance of observed versus expected heterozygosity; NS = not significant. Note: calculation of H_E in the captive population is not applicable (see text).